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Antimicrobial properties of three liverworts in axenic culture: *Blasia pusilla*, *Pallavicinia lyellii* and *Radula obconica*

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Abstract – Many liverworts have been reported to deter the growth of pathogenic agents such as fungi and bacteria. It has been generally assumed that the chemicals liverworts produce in their oil bodies are responsible for this activity. However, most of these studies have used plants from nature, and only a few of them have isolated and identified the specific compounds that are biologically active. This study aims to expand existing knowledge of biological activity of liverworts as follows: 1) testing whether plants raised in axenic culture display antimicrobial activity, 2) determining if antimicrobial activity can occur in taxa that lack oil bodies, and 3) isolating and structurally characterizing the compounds responsible for any activity observed. To this end, extracts from axenic cultures of *Blasia pusilla* L., *Pallavicinia lyellii* (Hook.) Carruth. and *Radula obconica* Sull. were tested for antimicrobial activity against *Bacillus subtilis*, *Escherichia coli*, and *Aspergillus niger*. *Blasia* retarded fungal sporulation, but displayed no inhibition of bacterial growth and *Pallavicinia* displayed only slight bacteriostatic activity against *B. subtilis*. *Radula* displayed the most pronounced antibacterial activity, especially against *B. subtilis*. These findings suggest that antimicrobial activity is more pronounced in taxa with oil bodies and confirm that bioactive compounds are still synthesized in axenic culture. The most active compounds in the fractions extracted from *Radula* are bibenzyls, compounds that are widespread in liverworts but of only sporadic occurrence in other plants.

Biological activity / *Blasia pusilla* / *Pallavicinia lyellii* / *Radula obconica* / bibenzyls / secondary metabolism

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INTRODUCTION

Liverworts are recognized as the basal, or first diverging lineage of land plants (Forrest *et al.*, 2006; Qiu *et al.*, 2006). They are morphologically and biochemically diverse, with three groups traditionally recognized based on habit, namely, leafy, complex thalloid, and simple thalloid. Approximately 90% of liverworts possess oil bodies (Crandall-Stotler & Stotler, 2000), distinctive organelles that arise from the endoplasmic reticulum (Suire, 2000). Although these may be of several different morphological types, in all cases they consist of a lipoprotein membrane that structurally resembles the tonoplast, surrounding a collection of lipid globules suspended in a protein matrix (Pihakaski, 1972; Crandall-Stotler & Stotler, 2000). Oil bodies have traditionally been considered organelles of essential oil and aromatic compound sequestration in liverwort cells and more recently have been demonstrated to be sites of isoprenoid synthesis (Suire *et al.*, 2000).

Liverworts exceed other bryophytes in their production of secondary metabolites, especially terpenoids and aromatic compounds (Mues, 2000). Eighty percent of the sesquiterpenes found in hepatics are enantiomers of those found in higher plants (Huneck, 1983; Asakawa, 2001), but there are also many novel terpene skeletons found in these lower plants (Mues, 2000). These secondary compounds lend liverworts peculiar biological activities, as detailed by Asakawa (1990; 1998) and Banerji (2001). For example, bicyclohumulenone, a highly valued compound used in the perfume industry, was isolated from *Plagiochila sciophila* Nees ex Lindenb. (Banerji, 2001: tab. 11); compounds in several species of *Frullania* Raddi cause contact dermatitis (Asakawa, 1990: tab. 25.4); and several species of liverworts produce compounds that display antitumor activity (Asakawa, 1990: tab. 25.2). Liverworts are also capable of resisting bacterial and fungal infection (Banerjee, 2001; Frahm, 2004) as well as deter feeding by insect larvae, slugs, snails and mammals (Asakawa, 2001). Several studies have demonstrated that compounds extracted from liverworts display antimicrobial activity, using disk diffusion assay methods (e. g., Banerjee & Sen, 1979; Mekuria *et al.,* 2005; Wang *et al.*, 2006; Zehr, 1993; Zhu *et al.*, 2006). However, only a few studies, such as those of Baek *et al.* (2004), Lorimer *et al.* (1993), Sauerwein & Becker (1990) and So & Chan (2001) have identified the specific compound(s) in the extracts that are responsible for this activity.

There are three objectives to this current study. The primary objective is to expand our knowledge of antibiotic and antifungal activity of the bryophytes, using axenically cultured samples of three representative taxa, namely, *Blasia pusilla* L., *Pallavicinia lyellii* (Hook.) Carruth. and *Radula obconica* Sull. *Blasia pusilla* is a basal member of the complex thalloid lineage (Forrest *et al*., 2006) and one of the few liverworts that does not possess oil bodies. It synthesizes several bis-bibenzyl dimers, but does not produce terpenoids (Asakawa, 2004). *Pallavicinia lyellii* is a phylogenetically advanced member of the simple thalloid lineage and one of the few liverworts that possesses hydrolyzed strands of "water-conducting" cells; it has an average of 10 intermediate-sized, botryoidal oil bodies per cell and is reported to produce sesquiterpenoids as major secondary metabolites (Asakawa, 1982). *Radula obconica* has one very large, segmented oil body per cell and is phylogenetically nested in the leafy liverwort lineage; it has not been previously studied, but other species of this genus are reported to elaborate bibenzyls and bis-bibenzyls but not terpenoids (Asakawa, 2004). The second objective is to determine if biological activity occurs only in taxa with oil

bodies, irrespective of the type of secondary metabolites produced; comparisons of the activity profiles of *B. pusilla* with those of the other two taxa will address this objective. The third is to isolate and structurally characterize the compound(s) responsible for any antimicrobial activity observed.

MATERIALS AND METHODS

Organisms

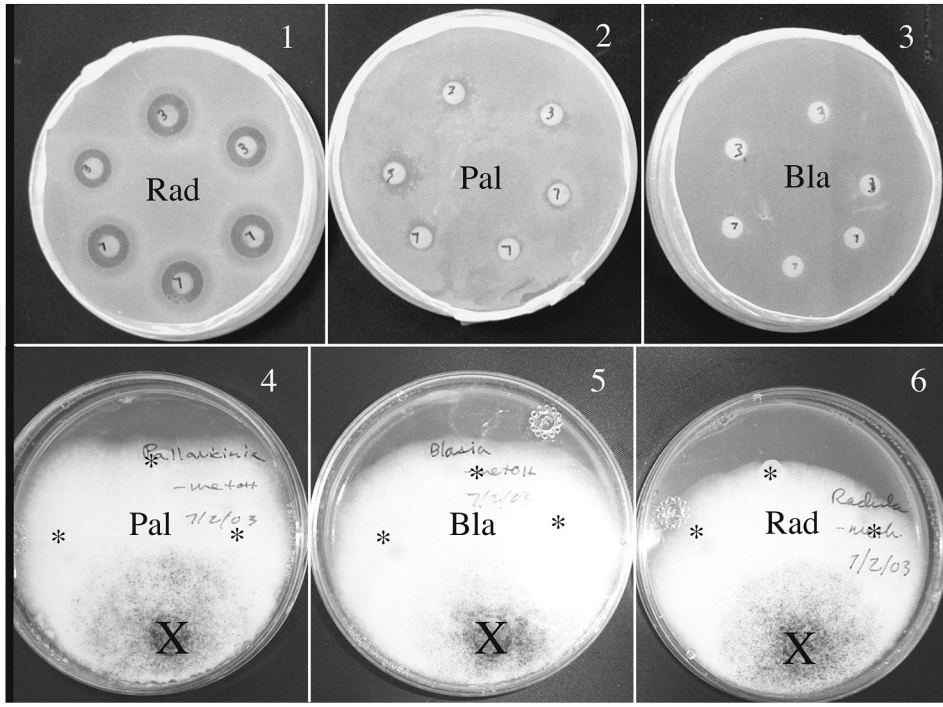
Axenically cultured specimens of *Blasia pusilla*, *Pallavicinia lyellii* and *Radula obconica* were tested for biological activity against two bacteria, the Gram$^+$, *Bacillus subtilis* and the Gram$^-$, *Escherichia coli*, and the fungus *Aspergillus niger*. The liverwort cultures were initiated from sporophyte capsules, using standard techniques (Hatcher, 1965), and were maintained in an environmental chamber under a 12h day/night cycle at 16°C, on Hatcher's media (Hatcher, 1965). Voucher collection details are as follows: *Blasia pusilla* L., Oregon, Josephine Co., nr O'Brien, Rough & Ready Creek, March 21, 1999, *Wheeler s.n.* (ABSH); *Pallavicinia lyellii* (Hook.) Carruth., Illinois, Jackson Co., Giant City State Park, March 1998, *J. Thompson s.n.* (ABSH); *Radula obconica* Sull., Illinois, Pope Co., Bell Smith Springs, Nov. 27, 1971, *Stotler 1513* (ABSH). Stock cultures of the bacteria, purchased from Carolina Biological Supply [*B. subtilis*, FR-15-4921; *E. coli* K-12 strain, FR-15-5065], were maintained on 1.5% agar LB® solidified broth at 30°C for *B. subtilis* and 38°C for *E. coli.* The fungus, also purchased from Carolina Biological Supply [*A. niger*, FR-15-5945], was maintained on Difco® potato dextrose agar at room temperature (approximately 25°C).

Bioactivity screenings

Initial screenings were done to determine which, if any, of the liverworts possessed antibacterial and/or antifungal activity. Two screenings were performed as follows: In the first screening, 1 g of axenic cultured plant material was extracted in either methanol or diethyl ether, resulting in both polar (methanol) and non-polar (diethyl ether) extracts. Since identical results were obtained with both solvents in the first screening, in the second screening, 1.9 g of axenic cultured plant material was extracted only in diethyl ether. The second screening was done only for the bacterial assay to see if a higher concentration of extract would yield different results.

The extraction procedure was modified from Asakawa (1988). Using a mortar and pestle, fresh, not dried, plant material was extracted with 10 ml of solvent for 7 days. The extract was then filtered through a 0.45 µm Millipore® filter to sterilize the extract. The solvent was evaporated to dryness and the extract was then stored at room temperature.

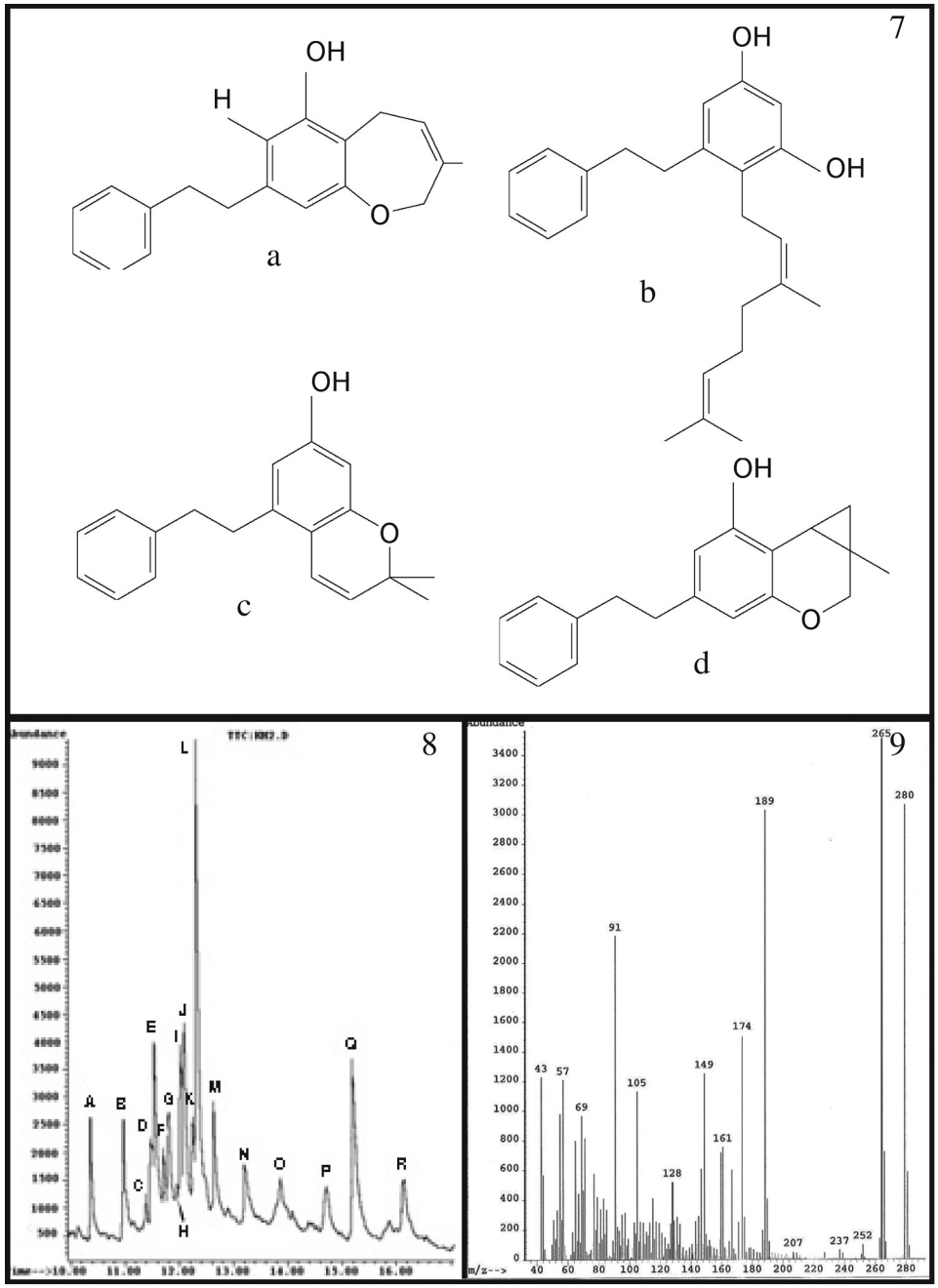
For the bacterial assay, 25 ml of agar-solidified LB broth was poured into a 15 mm diameter Petri dish. Bacterial cells were obtained from an overnight liquid LB broth suspension culture grown in a model R76 reciprocal water bath shaker and diluted to 6.72×10^6 cells/ml. This diluted solution of cells was added to 5 ml of autoclaved, cooled 0.8% agarose and then poured over the solidified LB media in the Petri dish to form a uniform bacterial lawn (Fig. 1-3).



Figs 1-6. Disk diffusion assays of antibacterial and antifungal activity shown by diethyl ether (**1-3**) and methanol (**4-6**) extracts against *Bacillus subtilis* (**1-3**) and *Aspergillus niger* (**4-6**) after 7 days of growth; 1 & 6 = *Radula obconica*, 2 & 4 = *Pallavicinia lyellii*; 3 & 5 = *Blasia pusilla.* In the antibacterial assays (1-3), the three test disks in the upper half of the plate are from 3-day extracts and the three in the bottom half are from 7-day extracts; zones of inhibition around the disks are seen as clear areas in the bacterial lawns. In the antifungal assays (4-6), X marks the inoculation plug, and * the position of the three test disks; note the less advanced growth of mycelia in 6 and the reduced zone of sporulation in 5.

For the fungal test, a plug of mycelial tips of *A. niger* was inoculated on Difco potato dextrose agar. The plug was obtained from a 1-week-old culture, using a sterile cork borer. Only the mycelial tips at the edge of the culture were used for the inoculum. The plug was placed near the side of the test dish (Figs 4-6).

The dried plant extracts were re-suspended in 1ml of their respective extraction solvents. Autoclaved paper disks, 7 mm in diameter, made of Whatman® No 5 filter paper, were individually soaked with 20 µl of the re-suspended extract. Control disks were similarly soaked in the extraction solvent. The disks were air-dried and then placed on the prepared bacterial or fungal test plates immediately after inoculation, five per bacterial plate and three per fungal plate. A disk soaked only with solvent was included on each plate as a control. Each set of tests was repeated three times, using newly cultured bacteria and fungi for each test replication. Each of the replicated sets of bacterial assays were monitored daily, with initial measurements of inhibition recorded after two days. The replicated fungal plates were monitored daily for 7 days. Inhibitory activity for the biological assays was measured as the diameter of the clear zones, where no microbial growth was evident. The diameter of the disk was included in the measurement of the clear zone.



Figs 7-9. Compounds identified in biologically active subfraction 9-AB of the diethyl ether extract of *Radula obconica*. **7.** Structural diagrams of the four compounds identified with GC/MS; a = radulanin A, b = prenyl bibenzyl, c = radulanin I, and d = a radulanin isomer. **8.** GC/MS chromatogram of subfraction 9-AB of *R. obconica*; the most abundant compound (peak L) is identified as radulanin A. **9.** MS chromatogram of peak L, identified as radulanin A.

Additional bioassays were performed to test whether extraction time or relative extract concentration would influence the degree of inhibition. To assay the effects of variation in extraction time, 1g samples of each taxon were extracted with diethyl ether for either three days or seven days. To assay the effects of variation in relative extract concentration, the 1ml solution of seven-day extract was diluted to 2 ml, 3 ml, and 4 ml. All extracts were processed and tested against *B. subtilis* as described above, except that three disks of each of the two extraction times were placed together on the bacterial plates instead of five disks of each (Figs 1-3). Again each experiment was repeated three times.

Compound isolation

To isolate the compound(s) responsible for the anti-microbial activity observed, 6.8 grams of fresh weight material of *R. obconica* was extracted in 20 ml of diethyl ether for 7 days. Ether extracts of *R. obconica* from both initial screening samples were combined with this extract and separated on a KC18F silica gel, 200 μm thick, thin layer chromatography (TLC) plate, with the developing solvent, 9:1 methanol:chloroform. The ten fractions obtained from this separation were designated by number, fraction 1 (F1) having the smallest R_f value and fraction 10 (F10), the largest. Each fraction was eluted from the plate, diluted to 1ml with diethyl ether, and then tested against *B. subtilis*, using the disk diffusion assay methods described above. Fraction 9, which showed the strongest and longest lasting inhibition of bacterial growth, was subsequently separated into subfractions using the same type of TLC and developing solvent as in the initial separation. The subfractions obtained from this separation, designated A through F, were also tested for activity against *B. subtilis*. Subfractions A and B, which produced similar results, were combined for GC-MS analysis as described below.

Chemical characterization of active compounds

The TLC fractions were initially tested for chemical composition using the following spray reagents: 30% H_2SO_4, plus heat, to test for sesquiterpenes (Asakawa, 1988), Dragendorff's reagent to test for heterocyclic N compounds (Harborne, 1998) and 10% methanolic KOH to test for anthraquinones (Harborne, 1998). In addition, intact plants were tested with several histochemical stains to determine the nature of the compounds in the oil bodies, including both a conc. H_2SO_4 test (Cappelletti *et al.*, 1986) and NADI reagent (Suire, 1970) to test for terpenes; a pink color was interpreted as a positive result in both systems. Other histochemical reagents used on the oil bodies included Sudan IV to detect lipids, ferric sulfate to detect tannins and IKI to detect starch (Jensen, 1962).

GC analysis of the 3-day and 7-day crude extracts of *R. obconica* was performed using a Shimadzu GC 20-10 with auto sampler. A 15 m Rtx-5, 0.25 mm i.d. crossbond 100% dimethyl polysiloxane capillary column was used with helium as the carrier gas to separate the components in a 1 μL injection. The temperature was 100°C at time of injection. The temperature was maintained at 100°C for 3 minutes then increased to 260°C, at a rate of 5°C/minute, and then held for 2 minutes.

The capillary gas chromatography/mass spectrometry (GC-MS) analysis of the combined 9 AB subfractions of the 7-day extract was carried out using a Hewlett-Packard Engine (Wilmington, DE) mass spectrometer system. Both electron impact and isobutane chemical ionization modes were utilized. The electron energy was set at 70eV with the ion source temperature maintained at

200°C. The individual components of the 1 μL injection were separated using a 15-meter DB-5 capillary column. The initial column temperature was set at 100°C (held for 0.3 minutes) and programmed to 260°C at 15°C per minute. The injector temperature was set at 270°C.

RESULTS AND DISCUSSION

Bioactivity screenings

In the first screening tests, similar results were observed for both the methanolic and the diethyl ether extracts of all taxa. With the methanolic extracts, however, the extract-soaked disks were difficult to dry completely prior to placement on the test lawns, and zones of inhibition were very irregular in outline because the extract diffused irregularly into the agar. Since similar patterns of bioactivity were observed with both types of extracts, all further screening tests used only the diethyl ether method of extraction.

All replications of the disk diffusion assay tests produced similar results. None of the liverworts displayed complete inhibitory activity against mycelial growth of *A. niger*, but *B. pusilla* partially inhibited sporulation and *R. obconica* retarded mycelial growth (Fig. 5, 6, Table 1). None of the liverworts completely inhibited the growth of *E. coli* and only in *R. obconica* was a zone of decreased bacterial density evident around the test disks. Even at the higher concentration of extract used in the second series of screenings, only *R. obconica* displayed complete inhibition of *B. subtilis. Pallavicinia lyellii* displayed some bacteriostatic activity against *B. subtilis*; however, the zone of inhibition was not completely clear of cells, but rather only showed decreased cell density (Fig. 2). So and Chan (2001) showed a low level of inhibitory activity of ethanolic extracts of *P. lyellii* against not only *B. subtilis*, but also *E. coli*. Both these and our results are, however, contrary to those of Banerjee and Sen (1979), who found that both aqueous and organic extracts of *P. lyellii* were completely inactive against these same organisms as well as seven other bacteria and two other fungi. These contrasting results may be related to the short, 36-hr extraction time used by Banerjee and Sen (1979), or perhaps there are multiple chemotypes in *P. lyellii*. *Blasia pusilla* showed no inhibitory activity against either *B. subtlis* or *E. coli.* The control disks soaked only with solvent likewise did not inhibit bacterial cell proliferation (Table 1).

Table 1. Antimicrobial activity of diethyl ether extracts from three liverworts as shown in disk diffusion assays. Bacterial inhibition is represented by the average diameter of the clear zone around the disk, including the 7 mm diameter of the disk; fungal inhibition is noted as differences in extent of the zone of sporulation and extent of mycelial advancement over the disks.

Liverworts/Microbes	Blasia pusilla	Pallavicinia lyellii	Radula obconica	*Diethyl ether control*
Bacillus subtilis ($Gram^+$)	No inhibition	Zone of less density, 0.85 cm	Clear zone, 2.0 cm	No inhibition
Eschericoli coli ($Gram^-$)	No inhibition	No inhibition	Zone of decreased cell density, 0.75 cm	No inhibition
Aspergillus niger	Moderate inhibition of sporulation	No inhibition	Retardation of mycelial growth	No inhibition

An important difference between our screening tests and those conducted by most other authors is that we used only plants from axenic culture as the source of our extracts. Plants from nature are often associated with fungal hyphae and/or surface bacteria that can themselves be a source of antimicrobial compounds as demonstrated by Opelt and Berg (2004). The use of cultured plants guarantees that any antimicrobial activity observed is due to compounds synthesized by the liverwort.

The most biologically active species in this study, *R. obconica*, possesses one very large oil body per cell, whereas *P. lyellii* possesses several small oil bodies per cell, and *B. pusilla* possesses no oil bodies. This might suggest that the compounds that are responsible for bacteriostatic activity are found in oil bodies; however, since *P. lyellii* showed only slight inhibition of bacterial growth, it cannot be assumed that presence of oil bodies necessarily imparts antibacterial activity. According to Suire *et al.* (2000), oil bodies are involved in terpene synthesis, but they can also sequester a wide variety of aromatic secondary metabolites that are synthesized elsewhere in the cell. Taxa without oil bodies may still synthesize compounds that are biologically active but these cannot be accumulated or concentrated as they are in taxa with oil bodies.

The differences seen in biological activity of these liverworts most likely are related to differences in their chemical constituents (Table 2). According to Asakawa (2004), *Pallavicinia subciliata* (Austin) Steph. produces complex labdane-type diterpenoids, but these are not found in *P. lyellii*. Such diterpenes have been demonstrated to have antibacterial activity (Ahmed *et al.*, 2004; Yang *et al.*, 2001). In our study two bands on a TLC plate of the whole extract obtained from *P. lyellii* tested positive for terpenes (Table 2). Asakawa (1982) has reported that sesquiterpenoids of the aromadendrane and cyclogermacrane groups are major components of *P. lyellii*. These terpenoids may be responsible for the slight inhibitory activity that *P. lyellii* extracts showed against *B. subtilis*.

Radula species are reported to produce characteristic bibenzyls (radulanins) and bisbibenzyls (Asakawa *et al.*, 1991). Fractions 9 and 10 of the *Radula* extract on the TLC plate turned dark blue, but none of its bands tested positive for terpenoids (Table 2). Four novel macrocyclic bis(bibenzyls), pusillatins A-D, along with riccardin C and F have been isolated from *B. pusilla* (Asakawa 2001; 2004; Hashimoto *et al.*, 1993). The bibenzyls and macrocyclic bis(bibenzyls) found in *Radula* species and *B. pusilla,* respectively, are likely responsible for the observed inhibitory activity against *A. niger*. In fact, previous

Table 2. Results of spray reagent tests for compound identification on TLC separated fractions of crude diethyl ether extracts of three liverworts. Extracts were separated with a 9:1 methanol:chloroform developing solvent; the number of fractions resolved for each taxon is indicated following the taxon name.

Liverworts/Reagents and compounds	R. obconica *– 10 fractions*	B. pusilla *– 7 fractions*	P. lyellii *– 7 fractions*
10% methanolic KOH test for anthraquinones	No color change	No color change	No color change
Concentrated sulfuric acid test for terpenes	Fraction 9 and 10 turned dark blue; results inconclusive	No color change	Fractions 4 and 6 turned red, + for terpenes
Dragendorf's Reagent test for alkaloids	No color change	No color change	No color change

research has shown that bis(bibenzyls) isolated from *Marchantia* L. do display antibacterial, antifungal, and cytotoxic activity (Keseru & Nogradi, 1995). Since *B. pusilla* is reported to contain compounds that typically are bacteriostatic, it is interesting that its extracts showed no antibacterial activity in our tests. In the absence of oil bodies, these compounds are probably not accumulated in the cells as they are in taxa with oil bodies; the lack of bacteriostatic activity may be due to inadequate amounts of the compounds in the extracts. Members of the Pallaviciniaceae have not been found to produce either bibenzyls or macrocylclic bis(bibenzyls) (Asakawa, 2004); therefore, the lack of fungal inhibitory activity exhibited by *P. lyellii* may be due to its production of sesquiterpenes rather than these unique stilbene derivatives found in the other two liverwort species used in this study. Our results agree with other studies that indicate that bryophytes display antibacterial activity more frequently against $Gram^+$ than $Gram^-$ bacteria (Asakawa, 1981). Whether the sustained inhibition of *B. subtilis* by *R. obconica* extracts is due to bibenzyls as suggested by Asakawa *et al.* (1991) is pursued in the succeeding sections on isolation and compound identification.

Compound isolation

Fractions 7, 8, and 9 from the first separation of *R. obconica* showed inhibitory activity but only fraction 9 was pursued for further study due to the ease of identification on the TLC plate, uniformity of inhibitory zone, and long lasting properties it displayed in the disk diffusion assay. The clear zones of inhibition were evident for more than 25 days. A and B subfractions from fraction 9 were studied further because they maintained the clear zone around the paper disk for over 25 days, indicating that the compound(s) responsible for this activity was stable and long lasting. The clear zones of inhibition produced by subfractions A and B were very similar (Table 3). Their GC and TLC profiles indicated that the same compounds were present in both non-polar (diethyl ether) subfractions, only in different proportions.

Chemical characterization of active compounds

As discussed above, reagent tests of the TLC plates were positive for terpenes only in *Pallavicinia lyellii*, and were negative for all other compounds tested (Table 2). Since sulfuric acid did give a color reaction with fractions 9 and 10 of the *Radula* extract, we attempted to test the oil bodies with it as well. However, as a histochemical test, it does too much damage to the cell to analyze subcellular localization of the fraction compounds. Although a 10% dilution of sulfuric acid did reduce cell damage, it did not cause a color reaction in the cells. The NADI reagent was negative for terpenes in all three taxa, but did give a positive test for the possible presence of cytochrome oxidase, indicated by a strong blue color (Jensen, 1962; Everson Pearse, 1972), in the rhizoids of *R. obconica*, and in its oil bodies. A similar positive test for cytochrome oxidase was also detected in some, but not all, of the oil bodies in *P. lyellii*, while *B. pusilla* showed no color reaction to the NADI reagent. The other histochemical tests for lipid, tannin, alkaloid, and anthraquinone localizations were negative in all taxa. Flegel and Becker (2000) have demonstrated that in *R. complanata* (L.) Dumort. a main bibenzyl component of methanol extracts, namely, 3-methoxybibenzyl, is, in fact, localized in the oil body, providing the first hard evidence that these aromatic compounds can be sequestered in oil bodies.

Table 3. Disk diffusion assays of antimicrobial activity of fractions and subfractions of a 7 day diethyl ether extract from *Radula obconica* against *Bacillus subtilis*. The fractions and subfractions were eluted from the TLC plates following separation with a 9:1 methanol:chloroform developing solvent. Inhibition is represented by the average diameter of the clear zone around the disk, including the 7 mm diameter of the disk. The confluent subfractions A and B were combined (9-AB) for further chemical characterization using GC/MS.

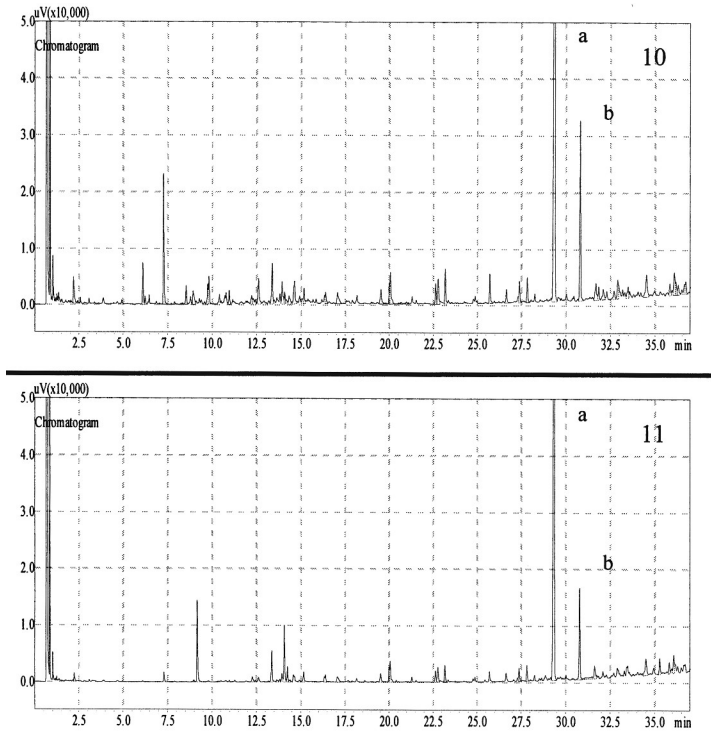
Fraction Number	*R_f values*	*Zone of Inhibition (cm)*
10	0.84	0
9	0.75	1.5
8	0.60	1.3
7	0.44	1
6, 5, 4	0.36, 0.27, 0.18	0
3, 2	0.14, 0.06	0
1	0.00	0
Subfractions of Fraction 9		
A	0.90	1.1
B	0.81	1.3
C	0.69	0
D	0.60	0
E	0.49	0.8
F	0.28	1.5

The GC/MS chromatogram of subfraction 9-AB is shown in Figure 8. Of the eighteen peaks labeled, eight, peaks A, B, E, J, M, N, O and P, are consistent with C_{22} – C_{29} hydrocarbons. However, these compounds are probably not responsible for the observed biological activity since such hydrocarbons can be broken down by microorganisms. The most abundant component of subfraction 9-AB is peak L. The electron impact mass spectrum of this peak, shown in Figure 9, has a molecular weight of 280 Daltons and is consistent with radulanin A (compound a in Fig. 7). Radulanin A has been found in natural populations of *R. complanata*, *R. constricta* Steph., *R. japonica* Gottsche ex Steph., *R. javanica* Gottsche and *R. tokiensis* Steph. (Takikawa *et al.*, 1989). Peaks C, G and H are molecular weight 280 Dalton radulanin isomers of peak L. Peak C is tentatively identified as the radulanin isomer, illustrated as compound b, Figure 7. Peak H is tentatively identified as radulanin I (see compound c, Fig. 7). Peak G is an unidentified radulanin isomer. Peak Q is tentatively identified as a prenyl bibenzyl compound having a molecular weight of 350 Daltons (compound d, Fig. 7). Peak R, having a molecular weight of 366 Daltons, appears to be an oxygenated isomer of peak Q. According to Asakawa *et. al.* (1991), radulanins and bibenzyl derivatives found in *Radula* spp. possess antimicrobial and antifungal properties. The presence of these radulanin-like compounds and prenyl bibenzyl derivatives most likely account for the observed antibacterial activity of subfractions A and B in this study. Finally, peak D is hexanededioic acid dioctyl ester, M.Wt 370, peak K is dibutylphthalate, M.Wt 278, peak I is bisdimethylbenzylphenol, M.Wt. 330, peak J is bisdimethylbenzyl tertiary butylphenol, M.Wt. 386 and peak F is an unknown compound with a molecular weight of 300 Daltons.

When tested against *B. subtilis*, the 7-day extract produced a slightly larger zone of inhibition as compared to the 3-day extract (Fig. 1, Table 4). In addition, the GC chromatograms of *R. obconica* indicate that differences in the chemical profiles of the 3-day and 7-day extracts do exist (Figs 10, 11). For example, the peaks

Table 4. Effects of extraction time and final extract concentration on the inhibition of *Bacillus subtilis* by diethyl ether extracts from *Radula obconica*, as measured by disk diffusion assays. Measurements were recorded at 2 days and 31 days after inoculation; inhibition is represented by the average diameter in cm of the clear zone around the disk, including the 7 mm disk diameter. Note that there was no decrease in the diameter of inhibition from day 2 to day 31 except for the 4-fold dilution of the initial 1 mL stock.

Time of Scoring/ Extraction Time	*2 days after inoculation*	*31 days after inoculation*
3 days	1.26	1.26
7 days	1.36	1.36
Time of Scoring/ Extract dilution		
1:2	1.15	1.20
1:3	1.13	1.13
1:4	1.10	1.06



Figs 10-11. GC chromatogram of 7-day (**10**) and 3-day (**11**) diethyl ether extracts of *Radula obconica*.

labeled "a" (retention time: 29.349 min.) and "b" (retention time: 30.789 min.) change substantially in peak area. Area of peak "a" from the 7-day extraction was 602252.6, but was 435099.5 from the 3-day extraction. Area of peak "b" from the 7-day extraction was 116421.8 and 32061.1 from the 3-day extraction. The area % of total did not change over time for peak "b" (8.3%), but decreased over time for peak "a" (57.7% for 3 day to 43.12% for 7 day). The longer extraction time resulted in larger amounts of crude extract from *R. obconica* and an increase in the number of compounds detected in the GC chromatogram. Surprisingly, a longer extraction time resulted in a decrease of crude extract from *P. lyellii*, as well as a smaller zone of bacteriostatic activity (Fig. 2), perhaps because of some degradation of the terpenes through time. There was relatively no change in the amount of crude extract from *B. pusilla*. From these results, it seems that the method of extraction of fresh material needs to be evaluated for each liverwort species and that a screening for biological activity or compound identification for some taxa should be done after 3 or fewer days of extraction.

The dilution assay indicated that there is a slight effect of concentration on the degree of inhibitory activity seen in *R. obconica*. The diameter of the zone of inhibition decreases from an average of 1.36 cm for the l ml dilution to 1.06 in the 1:4 dilution (Table 4).

CONCLUSIONS

Liverworts are an understudied group of land plants that have potential as a source of medicinal compounds. Although they are slow growing and do not produce a lot of biomass, they show great chemical diversity and are relatively easy to grow in axenic culture. Our study used only plants from axenic culture, confirming that the biologically active, secondary metabolites are synthesized by the liverwort and are not the products of bacterial or fungal symbionts commonly found in field populations. At a time that microbes are showing increased resistance to currently available drugs, the chemical diversity of liverworts offer hope for the discovery of novel compounds that can serve as models for the development of new pharmaceuticals. Furthermore, secondary compounds found in bryophytes have potential as natural, organic pesticides (Frahm, 2004; Mekuria *et al.*, 2005). In this study, the long-lasting biological activity of *Radula obconica* bibenzyls against *Bacillus subtilis* suggests a potential for these compounds to be used against other $Gram^+$ bacteria such as *Streptococcus* and *Staphylococcus*. The presence of large oil bodies in *R. obconica*, but not in *B. pusilla,* suggests that while oil bodies have an important role in the production and sequestration of biologically active metabolites (Flegel & Becker, 2000; Suire *et al.*, 2000), active compounds like the antifungal bis(bibenzyls) may be present even in the absence of oil bodies. Furthermore, some taxa with oil bodies, like *P. lyellii*, may show only moderate to no antimicrobial activity. It is not the presence of oil bodies that determines whether a taxon is able to inhibit microbial growth, but rather the nature of the secondary metabolites that are synthesized and sequestered by the plant.

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